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PRINCIPAL INVESTIGATOR: Jeffrey K. Griffith, Ph.D.

CONTRACTING ORGANIZATION: University of New Mexico Health Sciences Center
Albuquerque, NM 87131

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14. ABSTRACT

Purpose: The novel Concept that is being tested in this project is that allelic imbalance (AI) in tissue obtained at prostate biopsy can serve as a sensitive and independent marker for staging and predicting disease recurrence in prostate cancer. Scope: The two Aims of the project are to(i)determine whether the number of sites of AIin biopsy predicts pathological staging following prostatectomy and (ii) to determine the independence, positive and negative predictive values, sensitivity of AI in biopsy tissues as a prognostic marker for prostate cancer. Major Findings: The number of sites of AI has been measured in DNA from 131 prostatectomy tissues,102 tumor adjacent, histologically normal prostate tissues, 75 biopsy specimens with a positive diagnosis of prostate cancer, 22 tumor adjacent, histologically normal biopsy specimens and 11 normal prostate tissues from men without disease. The results demonstratethat AI is increased (i)in tumor and tumor-adjacent prostate tissues relative to normal prostate tissues, (ii) as a function of Gleason Sum and (iii)in recurrent prostate tumors. Significance: Allelic imbalance in prostate cancer tissues may provide a novel diagnostic and prognostic marker.

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INTRODUCTION

Autopsy studies demonstrate that 10-20 million men in the United States have undiagnosed prostate cancer, indicating that the vast majority of men with prostate cancer are asymptomatic throughout their lifetimes. However, the introduction of current screening modalities for prostate cancer, particularly the measurement of prostate-specific antigen (PSA) levels, have greatly increased the number of men referred for prostate biopsies and subsequently diagnosed with invasive prostate cancer. The choices of definitive therapy for most men with clinically localized disease are usually radical prostatectomy (RP) and radiation therapy (RT). The consequences and complications of these treatments are frequently severe, including incontinence, urethral stricture, impotence and death. Between 30 and 40% of men with clinically localized tumors choose RP accepting the associated morbidity and mortality because it offers the chance for cure. However, about 40% of clinically localized tumors treated with RP are subsequently determined extraprostatic on pathological examination, and may not be curable by RP alone. Thus, men with extraprostatic disease often suffer the morbidity of RP without curative benefit. Similarly, because current prognostic markers in prostate biopsy tissue cannot reliably differentiate tumors that will remain indolent from those that will rapidly progress, men receive RT or RP and experience their associated morbidity and mortality although, in many cases, the treatment is unnecessary and management with "watchful waiting" would be more appropriate. **Therefore, it is imperative to identify informative prognostic markers of prostate cancer progression that differentiate at the time of biopsy those men who will benefit from RT or RP from those who can be spared their expense, consequences, risks and reduced quality of life.**

We have shown previously that the content of telomere DNA ("TC", a surrogate marker for telomere length) is an independent predictor of disease-free survival in prostate cancer. We hypothesize that (i) critically shortened telomeres generate genomic instability and thus, phenotypic variability in neoplastic prostate tissues, and (ii) this promotes the genesis of lethal, metastatic tumor cells. ***A testable prediction of this hypothesis, and the focus of this proposal, is that allelic imbalance (a reflection of genomic instability) in prostate biopsy tissue predicts staging and future disease recurrence.*** To test this proposition, we have developed a multiplex, PCR-based method for assessing allelic imbalance (AI), a measure of genome instability, at sixteen non-linked microsatellite loci in the genome. The method uses commercially available primers, reagents, instrumentation and analysis software, and is suitable for analysis of fresh, frozen or paraffin-embedded archival tissues. The AI assay requires only 1-2 ng of DNA, does not require paired normal tissue, can be performed on tissue containing mixtures of tumor and normal cells, and therefore is particularly well-suited for prognostic assessment of prostatic biopsies.

BODY

Tasks: The agreed upon specific aims and tasks to be completed under the Hypothesis Award are as follows:

Aim One: *To determine whether the number of sites of allelic imbalance (AI) in biopsy predicts pathological staging following prostatectomy.* This will be accomplished by assessing the association between AI in prostate biopsy with the pathological stage of the patients' paired prostatectomy specimen. The tasks associated with this aim are:

Task 1: Purify DNA from approximately 300 archival specimens of prostate biopsy and prostatectomy tissues.

Task 2: Measure AI in DNA from the 300 archival specimens of prostate biopsy and prostatectomy tissues.

Task 3: Determine the association between AI in prostate biopsy with the pathological stage of the paired prostatectomy specimen.

Aim Two: *To determine the independence, positive and negative predictive values, sensitivity (i.e. frequency of false-positives) and specificity (i.e. frequency of false-negatives) of AI in biopsy tissues as a prognostic marker for prostate cancer*. This will be accomplished by associating the number of sites of AI in prostate biopsy with patients' recurrence data. The task associated with this aim is:

Task 4: Using data from Task 2, determine the association between AI in prostate biopsy and patient outcome.

Progress Relative to Tasks:

Task 1: Purify DNA from approximately 300 fresh and archival specimens of prostate biopsy and prostatectomy tissues.

We have purified DNA from the following **341** archival prostate tissues:

- 131 prostatectomy tissues
- 102 tumor adjacent, histologically normal prostate tissues
- 75 biopsy specimens with a positive diagnosis of prostate cancer
- 22 tumor adjacent, histologically normal biopsy specimens
- 11 specimens of normal prostate tissue from men without disease

Task 2: Measure AI in DNA from the 300 archival specimens of prostate biopsy and prostatectomy tissues.

We have measured AI in all of the **341** specimens listed above.

Task 3: Determine the association between AI in prostate biopsy with the pathological stage of the paired prostatectomy specimen.

We first analyzed AI as a function of tissue source: normal, coexisting histologically normal (CHN) tissue from biopsy, tumor containg tissue from biopsy, CHN from prostatectomy and tumor containg tissue from prostatectomy. As shown in Figure 1, the 2-sided nonparametric Kruskal-Wallis Test indicates a highly significant increase in the mean number of sites of AI in all tumor or CHN tissues, either from biopsy or prostatectomy, to relative to normal prostate tissues from autopsies.

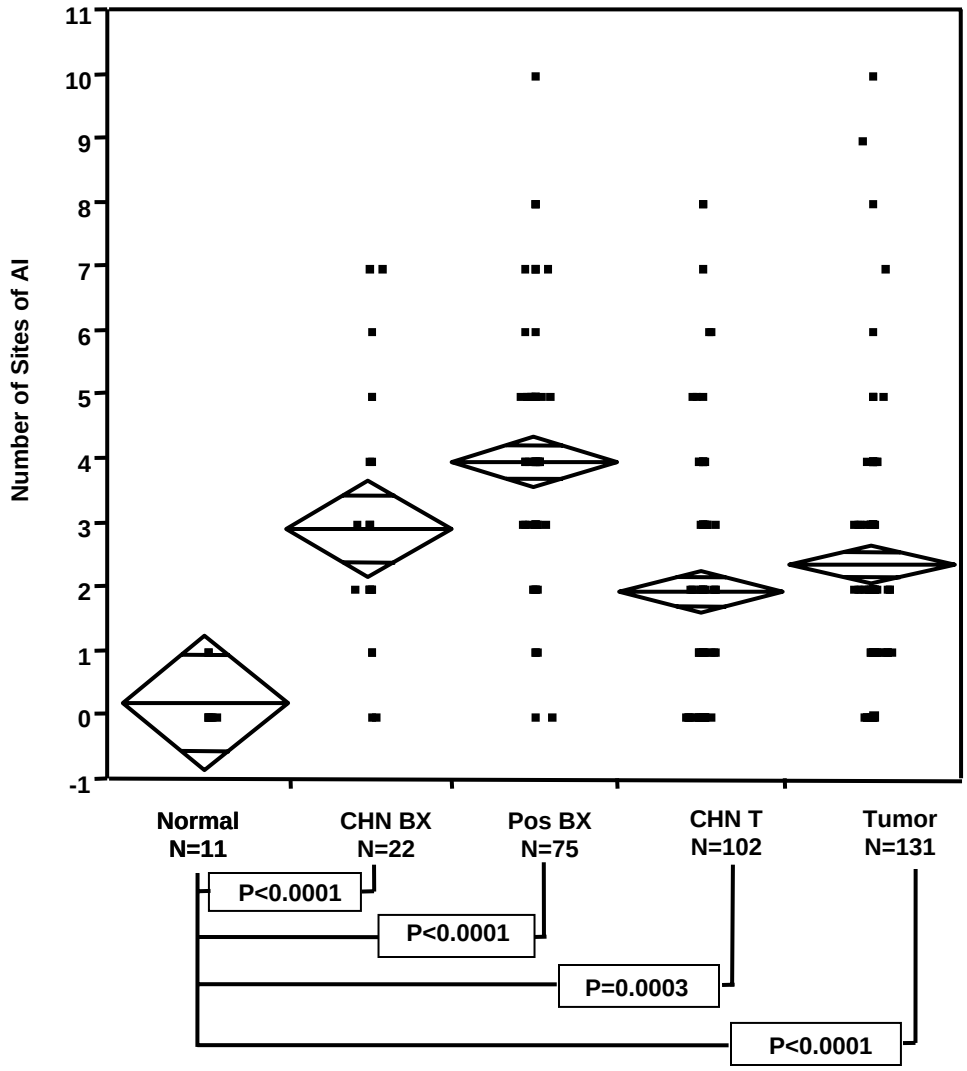

Figure 1. Numbers of sites of allelic imbalance (AI) in normal, tumor-adjacent, prostatectomy and biopsy tissues. The number of sites of AI is shown on the y-axis. The sources of the tissues and number of samples in each group (N) are shown on the x-axis. The line across each diamond represents the group mean. The height of each diamond represents the 95% confidence interval for each group. Statistical significance (p) was determined using the 2-sided nonparametric Kruskal-Wallis test. Although the data points are horizontally shifted, some are still overlapping, and therefore not visible. See text for additional details.

We next evaluated the association between AI and Gleason Sum. Tumor tissues were stratified by the Gleason Sum as follows: "Low" Group: Gleason Sum 3-6 (N=52), "High" Group: Gleason Sum >6 (N=99). As shown by logistic regression (Figure 2A), there was a highly significant association between AI and Gleason Sum (p=0.007). A similar result was obtained when the data were analyzed by nonparametric Wilcoxon Rank Sums test (p=0.017).

Similarly, logistic regression and Wilcoxon Rank Sums tests (not shown) indicate significant associations between AI and Gleason Sum (p=0.026, 0.017, respectively) when Gleason Sums are stratified into three groups: 3-6 (N=52), 7 (N=85) and >7 (N=17).

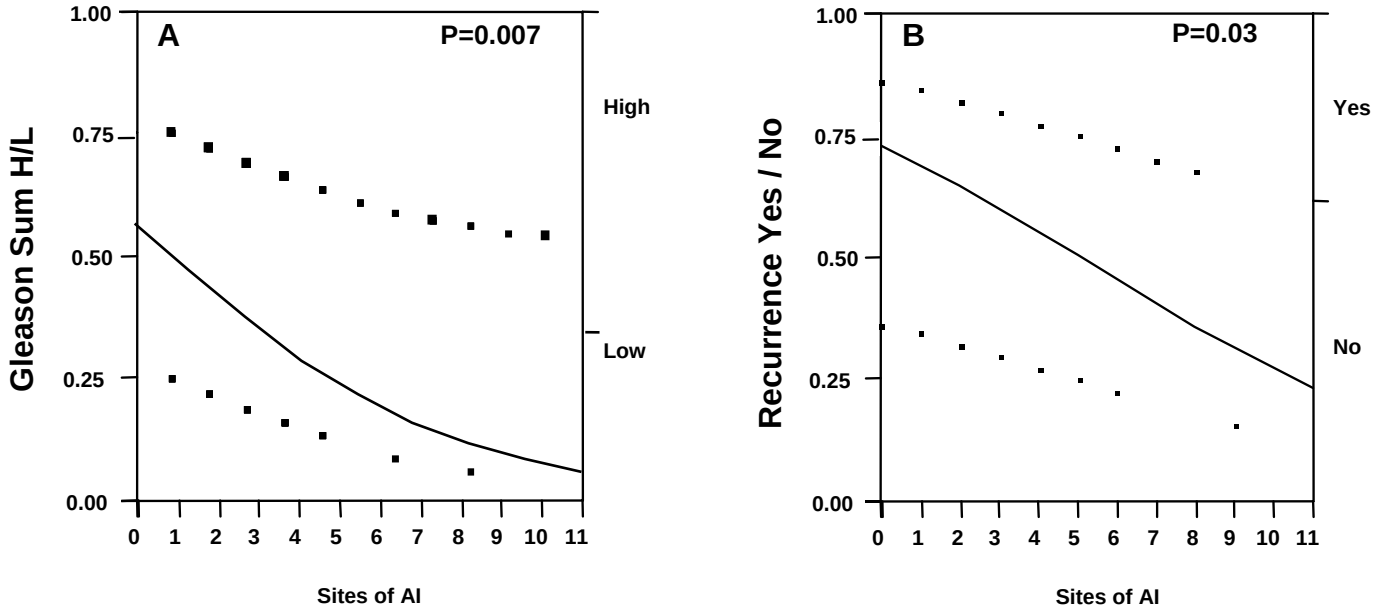

Figure 2. Associations between number of sites of allelic imbalance (AI) and Gleason sum score and five year prostate cancer-free survival. The relationships between AI and Gleason Sum (panel A), and prostate cancer-free 5-year survival (panel B) were evaluated by logistic regression. Logistic regression estimates the probability of choosing one of the specified parameters (e.g. low vs. high Gleason Sum) as a function of AI. In a logistic probability plot, the y-axis represents probability. The number of sites of AI is shown on the x-axis. The proportions of tumors with "low" Gleason Sum (i.e., 2-6, n= 50) and "high" Gleason Sum (i.e., >6, n=99), and the proportions from recurrence-free (n=74) and recurrent tumors (n= 46) for each value of AI are shown on the y-axes of panels A and B, respectively. Recurrence was defined as distant metastasis, biochemical recurrence (i.e. rising PSA), or death as a consequence of prostate cancer.

In summary, these data indicate that: (i) the number of sites of AI in tumors, biopsies and tumor adjacent prostate tissues is significantly greater than the number of sites in normal tissues, demonstrating potential diagnostic value, (ii) the number of sites of AI in biopsy tissue is at least as great, and likely greater, than the number of sites in prostatectomy tissues and (iii) the number of sites of AI is associated with Gleason Sum. ***Taken together, the results are consistent with the conclusion that AI in biopsy tissues predicts Gleason Sum in tumors.***

Task 4: Using data from Task 2, determine the association between AI in prostate biopsy and patient outcome.

At least 5 years of follow up data was available for 120 men in the cohort. Recurrence was defined as distant metastasis, biochemical recurrence, i.e. rising PSA, or death as a consequence of prostate cancer. As shown by logistic regression (Figure 2B), there was a highly significant association between AI and recurrence (p=0.03). Using the data in Figure 2, we postulated that AI thresholds of >1 and >4 could be used to predict Gleason Sum and disease recurrence, respectively. As shown in Figure 3, there was a highly significant association between AI stratified by these thresholds and both Gkeason Sum (p=0.003) and five-year recurrence-free survival (p=0.004).

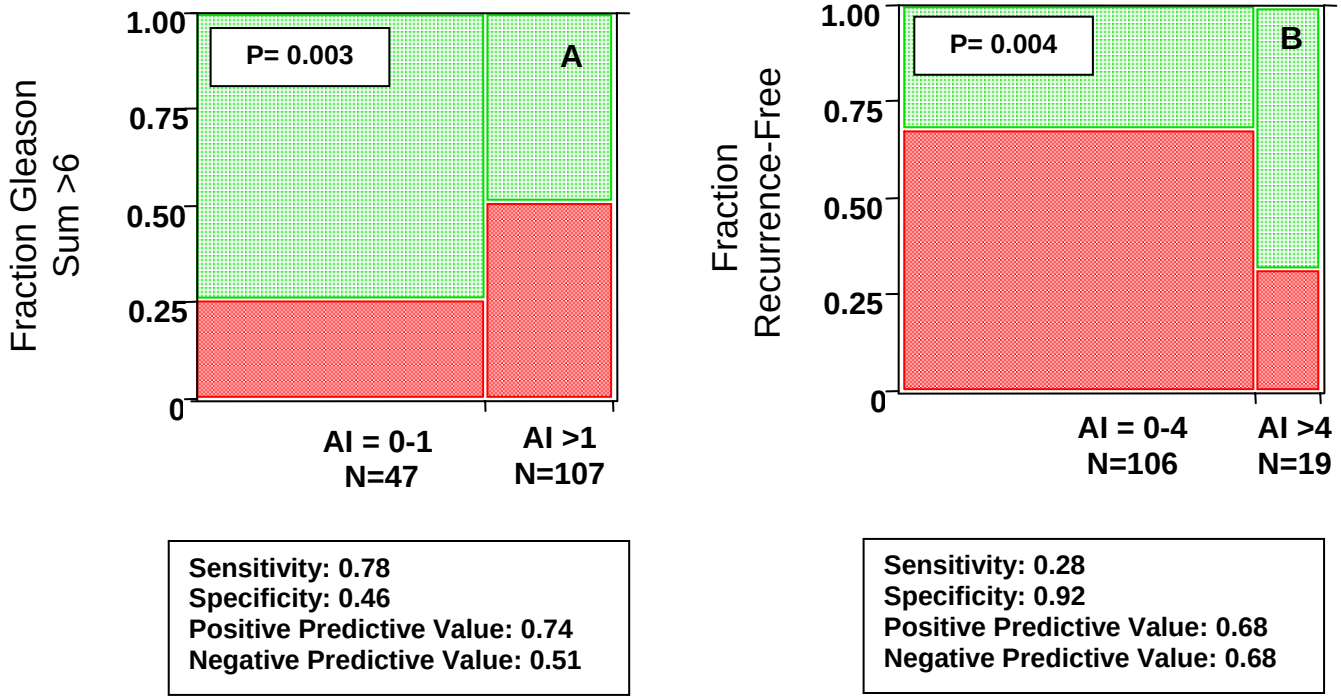

Figure 3. Associations between number of sites of allelic imbalance (AI) and Gleason sum score and five year prostate cancer-free survival. The y-axes show the fraction of samples with Gleason Sum >6 (panel A) or 5 year recurrence-free survival (panel B).The AI groupings are shown on the x-axis. The number of samples in each group (N) is shown on the x-axis. Statistical significance (p) was determined using Fisher's Exact Test. Sensitivity, specificity, positive predictive value and negative predictive values are shown in the boxes. See text for additional details.

Follow up data was also available for a small number (N=15) of biopsy specimens. Despite the very small sample size, the same relationship between the number of sites of AI and patient is clear (Figure 4) and approached statistical significance (p=0.08).

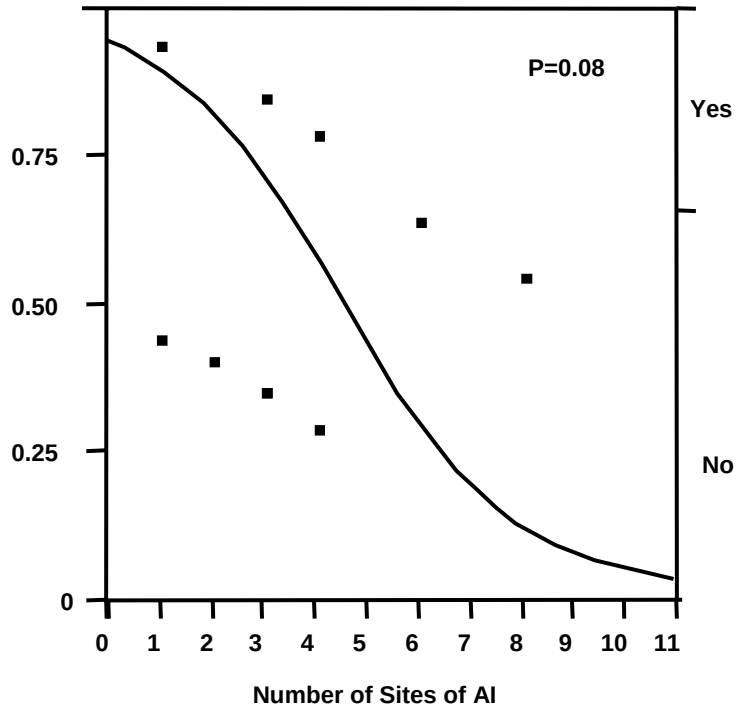

Figure 4. Associations between number of sites of allelic imbalance (AI) in biopsy tissue and five year prostate cancer-free survival. The relationships between AI and prostate cancer-free 5-year survival evaluated by logistic regression as in Figure 2B. The number of sites of AI is shown on the x-axis. The proportion of recurrence-free (n=5) and recurrent prostate cancers tumors (n=10) for each value of AI are shown on the y-axis. Recurrence was defined as distant metastasis, biochemical recurrence (i.e. rising PSA), or death as a consequence of prostate cancer.

In view of the similarity in AI in tumors and biopsies, and the similarity in the association between AI and patient outcome in tumors and biopsies, we conclude that the results are consistent with the conclusion that AI in biopsy tissues predicts patient outcome. A blinded investigation with 100 biopsy specimens with 10 years of follow up data is ongoing and will be completed within the next 60 days.

Additional Studies: To validate the operationally-defined threshold for AI, we measured the allelic ratios for 1382 heterozygous loci in an independent validation set comprised of normal samples consisting of specimens from bone (n=2), breast (n=10), buccal (n=53), lymph node (n=5), peripheral

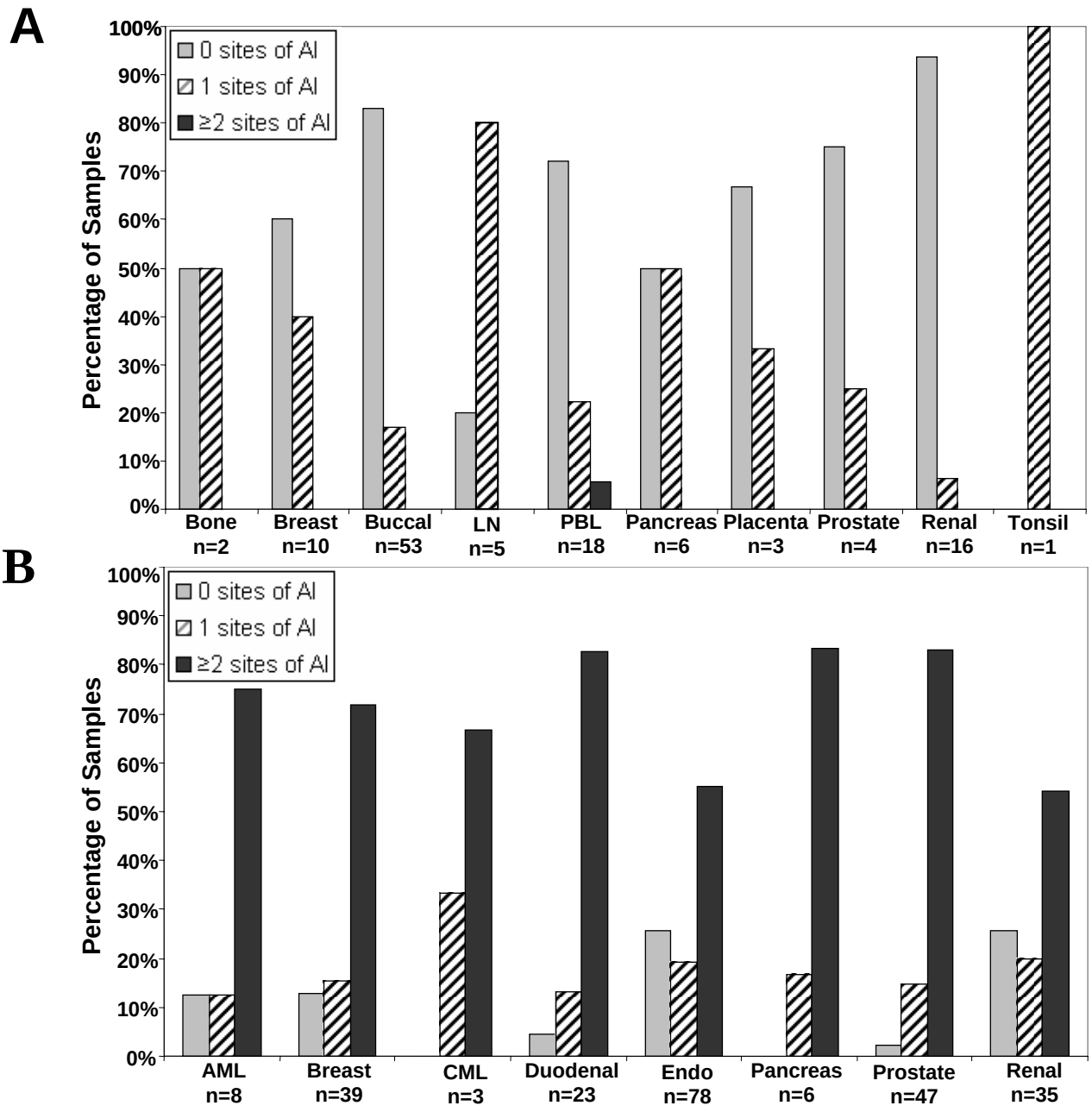

Figure 5. Frequency of allelic imbalance in normal and tumor tissues. The numbers of sites of allelic imbalance (i.e. 0, 1, ≥ 2) were determined in 118 samples of normal tissue and in 239 samples of tumor tissue. The number of specimens in each tissue set (n) is indicated below the set designation. Abbreviations: Lymph Node: LN; Peripheral Blood Lymphocytes: PBL; Acute Myelogenous Leukemia: AML; Chronic Myelogenous Leukemia: CML; Endometrial: Endo.

blood lymphocytes (n=18), pancreas (n=6), placenta (n=3), prostate (n=4), renal (n=16) and tonsil (n=1) tissues. As shown in Figure 5A, 88 (74.6%), 29 (24.6%), and 1 (0.8%) of the 118 normal tissues specimens contained 0, 1 and 2 loci with AI, respectively. In this validation set of normal tissues, only 32 sites, out of 1382 possible sites, of AI were detected, thus representing an incidence of 0.27 sites of AI per sample.

The frequency of AI also was determined in 2792 heterozygous loci in a test set comprised of frozen or paraffin-embedded specimens of AML (n=8), breast (n=39), CML (n=3), duodenal (n=23), endometrial (n=78), pancreatic (n=6), prostate (n=47), and renal (n=35) cancers. As shown in Figure 5B, 37 (15.5%), 41 (17.2%), and 161 (67.4%) of the 239 tumor tissues specimens contained 0, 1 and $\geq$ 2 loci with AI, respectively. In contrast to the normal tissues, 611 sites of AI were detected, thus representing a mean incidence of 2.56 unbalanced loci per sample, nearly 10 times greater than the frequency in the normal tissues ($p<0.0001$). Finally, we compared the incidences of AI observed in the 118 normal tissue specimens and 239 tumor specimens in the test set to the incidences in a second, completely independent validation set comprised exclusively of prostate tissues. Nine (90%) and 1 (10%) of the 10 normal prostate tissues contained 0 and 1 loci with AI, respectively. In contrast, 15 (8.6%), 36 (20.6%%), and 124 (70.9%) of the 175 tumor specimens contained 0, 1 and $\geq$ 2 loci with AI, respectively. We also measured AI in 119 specimens of coexisting histologically normal (CHN) prostate tissues from sites outside the tumors' margins. Importantly, 28 (23.5%), 26 (21.8%%), and 65 (54.6%) of the CHN specimens contained 0, 1 and $\geq$ 2 loci with AI, respectively, similar to the frequencies in both prostate tumors (Figure 5B).

KEY RESEARCH ACCOMPLISHMENTS

- Purified and quantitated genomic DNA from 341 prostate tissues, 192 non-prostate tumors and 114 normal tissues.
- Measured extent of allelic imbalance (AI) in 341 prostate tissues, 192 non-prostate tumors and 114 normal tissues.
- Demonstrated that AI is increased in tumor and tumor-adjacent prostate tissues.
- Demonstrated that AI increases as a function of Gleason Sum.
- Demonstrated that AI is increased in recurrent prostate tumors.
- Determined the positive and negative predictive values, sensitivity and specificity of AI in biopsy tissues as a predictor of both Gleason Sum and disease recurrence
- Demonstrated that AI is increased in cancers from multiple organ sites.

REPORTABLE OUTCOMES

- A database has been produced that contains AI information for 341 prostate tissue specimens and their associated, anonymous patient histories, including age at diagnosis, treatments, Gleason Sum, tumor stage, pelvic node and seminal vesicle involvement, grade, size, length of disease free survival or date and cause of death and diagnosis.
- Five presentations at national meetings (abstracts are listed below)
- One submitted paper accepted contingent on revisions describing the AI technology. A second manuscript will be submitted within the next 3 months.
- Grant submissions (both pending review) to the NCI and the DOD PCRP to extend these investigations in prospectively studies
- A patent disclosure has been filed for the use of the allelic imbalance assay in cancer diagnosis and prognosis.
- Research opportunities for four graduate and two undergraduate students.

Financial Support:

Jeffrey Griffith, Ph.D.	Principal Investigator
Ming Ji	Technician
William Hines	Graduate Student (Ph.D., 2005)
Alexandra Fajardo	Undergraduate Student (B.S., 2005)

Research Opportunities:

Christopher Heaphy	Graduate Student
Christina Haaland	Graduate Student
Kimberly Butler	Graduate Student
Jessica Wyaco	Undergraduate Student (B.S., 2005)

Published and Submitted Papers:

1. Christopher M. Heaphy, William C. Hines, Kimberly S. Butler, Christina M. Haaland, Glenroy Heywood, Edgar Fischer, Marco Bisoffi and Jeffrey K. Griffith, Measurement of Genome-wide Allelic Imbalance in Human Tissue Using a Multiplex PCR System, Cancer Epidemiology, Biomarkers and Prevention, Accepted, contingent on revisions

Abstracts:

1. J. Wyaco, C. M. Heaphy, M. Bisoffi and J.K. Griffith (2005) Telomere DNA Content and Allelic Imbalance in Normal, Tumor-adjacent Histologically-normal, and Tumor Prostate Tissue. 2005 FASEB Experimental Biology Meeting, San Diego, CA.

2. C.M. Heaphy, M. Bisoffi, C. A. Fordyce, C.M. Haaland-Pullus, W.C. Hines, N.E. Joste and J.K. Griffith (2005) Telomere DNA Content and Allelic Imbalance in Histologically Normal Tissue Adjacent to Breast and Prostate Tumors: Implications for Prognosis. 2005 Minority Trainee Research Forum, Nationial Institutes of Health, Bethesda, MD.

3. C.M. Heaphy, C.A. Fordyce, N.E. Joste, A.Y. Smith, W.C. Hunt and J.K. Griffith (2005) Association Between Cancer-free Survival and Telomere DNA Content in Prostate Tumors. RPMI Conference, Prostate Cancer: Roadmap to the Future, Niagra Falls, NY.

4. C. M. Heaphy, M. Bisoffi, C.A. Fordyce, A. Mangalik and J.K. Griffith (2005) Telomere DNA Content and Allelic Imbalance in Histologically-normal Tissue Adjacent to Breast Tumors. 2005 DOD Era of Hope Symposium, Philadelphia, PA.

5. C.M. Heaphy, M. Bisoffi, C.A. Fordyce, C.M. Haaland-Pullus, W.C. Hines, N.E. Joste and J.K. Griffith (2005) Telomere DNA Content and Allelic Imbalance Predict Disease-free Survival and Define Field Cancerization in Histologically Normal Tissue Adjacent to Breast Tumors. San Antonio Breast Cancer Symposium. San Antonio, TX.

CONCLUSIONS

- Based on the data generated with the support of this award, we postulate that the number of sites of AI in prostate biopsy tissue: (i) predicts Gleason Sum, (ii) predicts prostate cancer recurrence and (iii) is diagnostic of coexisting cancer.

- The novel finding that AI is similar in cancerous and CHN prostate tissue predicts that AI determination will not be confounded by CHN tissue in the biopsy, and suggests that the AI assay may provide useful diagnostic information even if the needles miss histologically transformed cancer tissue completely.

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